Role of Accessory DNA Polymerases in DNA Replication in Escherichia coli: Analysis of the dnaX36 Mutator Mutant

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The dnaX36(TS) mutant of Escherichia coli confers a distinct mutator phenotype characterized by enhancement of transversion base substitutions and certain (−1) frameshift mutations. Here, we have further investigated the possible mechanism(s) underlying this mutator effect, focusing in particular on the role of the various E. coli DNA polymerases. The dnaX gene encodes the τ subunit of DNA polymerase III (Pol III) holoenzyme, the enzyme responsible for replication of the bacterial chromosome. The dnaX36 defect resides in the C-terminal domain V of τ, essential for interaction of τ with the α (polymerase) subunit, suggesting that the mutator phenotype is caused by an impaired or altered α-τ interaction. We previously proposed that the mutator activity results from aberrant processing of terminal mismatches created by Pol III insertion errors. The present results, including lack of interaction of dnaX36 with mutM, mutY, and recA defects, support our assumption that dnaX36-mediated mutations originate as errors of replication rather than DNA damage-related events. Second, an important role is described for DNA Pol II and Pol IV in preventing and producing, respectively, the mutations. In the system used, a high fraction of the mutations is dependent on the action of Pol IV in a (dinB) gene dosage-dependent manner. However, an even larger but opposing role is deduced for Pol II, revealing Pol II to be a major editor of Pol III mediated replication errors.

The mechanisms by which cells produce mutations, or try to avoid making them, are of significant research interest. Mutations may occur from replication errors, as DNA replication proceeds with high but not infinite accuracy. While the fidelity of individual DNA polymerases, including their base insertion fidelity and proofreading ability, has been investigated in detail (for reviews, see references 43 and 44), recent emphasis has shifted to the fidelity of the chromosomal replisomes, the multisubunit complexes that perform the simultaneous replication of leading and lagging strands. Specific issues of interest are the contribution of the various replisomal subunits, the mechanisms underlying the differential fidelity of leading and lagging strand replication, and the involvement of the additional DNA polymerases that have been discovered in recent years.

In the model system Escherichia coli, chromosomal replication is performed by the 17-subunit protein complex DNA polymerase III (Pol III) holoenzyme (HE) (49, 50, 56). HE is organized into several functional modules: two Pol III core units (one for each strand), two β-clamp processivity factors, and the DNA complex. Each Pol III core is made up of three subunits (α, ε, and θ), in which α is the DNA polymerase, ε is the proofreading subunit (3′→5′ exonuclease), and θ is a stabilizing factor for the ε subunit (37, 82). Each β-clamp is a dimer of identical subunits (B2) in the shape of a ring, tethering the core units to the DNA through an interaction with the α subunit. This interaction with the β-clamp is responsible for high processivity of DNA synthesis, which is particularly important for synthesis of the leading strand (38). The seven-subunit DnaX-complex (τγδεγψ) contains an ATP-powered clamp-loader activity (γδεψ) responsible for assembly of the β clamps onto DNA, which needs to be done repeatedly on the lagging strand. The τψ component of the DnaX complex serves essentially as an organizing center of HE. Each τ subunit interacts with the α subunit of a Pol III core, coupling the leading- and lagging-strand polymerases (22, 34, 49, 51, 56, 77) and also binds the γδεψ clamploading complex (49, 56, 57). In addition to being the structural scaffold of HE, τ also plays important regulatory roles. For example, it interacts with the DnaB helicase, regulating the speed of the replication fork (10, 21), and triggers the dissociation of the lagging-strand polymerase at the end of Okazaki fragments through its interaction with the α subunit (45, 46, 51). Recently, an HE containing three τ subunits (instead of two τ and one γ) and three Pol III core molecules has been described and may be a biologically relevant species (50).

In the present study, we were particularly interested in the role of τ subunit in the control of chromosomal replication fidelity. The τ protein is the 71.1-kDa (643 amino acids) full-length product of the dnaX gene. The gene also produces the 47.5-kDa γ subunit, which results from an early termination in the dnaX reading frame through a programmed −1 ribosomal frameshift. As a result, γ is identical to the first 430 residues of τ (3, 16, 85). The τ subunit contains five distinct domains, I through V, of which domains I to III correspond to γ, whereas domains IV and V are unique to τ (21–23). Domain IV medi-
ates the interaction of τ with the DnaB helicase (21), while domain V is responsible for the α-τ interaction. Since τ is present as a dimer (or trimer), the α-τ interaction effectively couples two (or three) Pol III core assemblies within the HE, thus facilitating joint synthesis of leading and lagging strands. The α-τ interaction is also responsible for the proper cycling of Pol III on the lagging strand (22, 45, 46, 50).

Previously, our laboratory has investigated the properties of certain E. coli dnaX mutants (61). These studies revealed that the temperature-sensitive allele dnaX36, encoding a mutant τ subunit containing a Glu-to-Lys change at residue 601 in domain V (4), displayed elevated mutation rates (61). This mutator phenotype suggested that one additional function of τ is to promote the fidelity of the chromosomal replication complex. Interestingly, the mutator effect is characterized by an unusual specificity: transversion base substitutions and (−1) frameshifts in non-run sequences are specifically enhanced. This observation led us to propose a model in which the τ subunit is important for the proper response of HE after certain misincorporation events by α subunit (61). In this model, certain transversion mismatches provide temporary stalling points for HE, which require the action of τ subunit to be resolved in an error-free manner. In the absence of proper α-τ interaction, these mismatches are processed in an error-prone manner, either as transversion base substitutions (by direct extension) or as (−1) frameshifts by misalignment-extension in sequence contexts where the misinserted base is complementary to the next template base. The latter type of misalignment-extension has been observed for many polymerases and represents a preferred way of HE under these circumstances (61).

In the present study, we have further investigated the role of the τ subunit as a fidelity factor. Our focus was on the source of the replication errors occurring in a dnaX36 strain and on the role of the additional E. coli DNA polymerases (Pol I, II, IV, and V), which have been increasingly considered to be potentially important players in various aspects of replication and mutagenesis (1, 17, 20, 28, 35, 41, 50).

MATERIALS AND METHODS

Media. Solid and liquid media have been described previously (14). Minimal medium (MM) was supplemented with 0.4% glucose or lactose as a carbon source, 5 μg of thiamine/ml and, where appropriate, 50 μg of kanamycin/ml. Solid medium contained 1.5% agar (Difco). The XPG plates used for papillation assays are minimal medium plates containing glucose (0.2%), phenyl-β-D-galactopyranoside (P-Gal) (0.5 mg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) (14). Where required, antibiotics were added at the following final concentrations: chloramphenicol, 20 μg/ml; kanamycin, 25 μg/ml; rifampin, 100 μg/ml; tetracycline, 12.5 μg/ml; spectinomycin, 50 μg/ml; and streptomycin, 50 μg/ml.

Strain constructions. The strains used in the present study are listed in Table 1. All strains used for the mutagenesis experiments are derivatives of KA796 (ara thi araD [Apob]) (71). Derivative strains were constructed by P1 transduction using PivτA and/or F′ crosses introducing F′proloc from strains CC102, CC104, CC105, and CC106 (9). NR13183 is a trpE9777 derivative of KA796 containing the trpE9777 marker (75) derived from NR3951 (72). The dnaX36 allele was introduced either from strain NR12159 based on the close linkage (90%) with the trpE9777 based on linkage with nearby srl kanamycin resistance. The strains NR9163 and NR9559, respectively (14, 69), selecting for tetracycline or kanamycin resistance. The recA56 allele was transferred from strain UTH2 (89) based on linkage with nearby ara Tn10, selecting for tetracycline resistance, followed by testing for UV sensitivity. The Apob trpE9777 allele was transferred by transduction from strain SH2101 (11) using spectinomycin resistance as a selective marker, followed by checking for acquired streptomycin resistance. Strains carrying the polBex1 allele were constructed as described previously (1) by first converting the recipient strain to leu−:Tn10, followed by conversion to leu− polBex1 using as a donor strain HC101 (polBex1 leu−) (18). The presence of the polBex1 allele was confirmed by backcrossing into a dnaX36 mutL strain containing the F′proloc from strain CC104 and scoring for hyper-papillation on X-Gal plates. The dnaX36 polBex1 combination leads to a very strong mutator phenotype (see Results), which is readily visualized on these plates. The polA1 allele was transferred by transduction from strain MHH210 (25) using linkage with transposon zyg-261:Tn10. The ΔmutD::cat allele was transferred from strain RW82 (91), selecting for chloramphenicol resistance. The ΔmutR::kan allele was transferred from strain YQ7207 (55) using selection for kanamycin resistance. The presence of the deletion was confirmed by PCR. The dnaQ49 and dnaQ930 alleles were transferred from strains NR9605 (70) and NR9986 (81), respectively, using linkage with transposon insertion zac-502:Tn10, selecting for tetracycline resistance and checking for mutator phenotype or temperature sensitivity. The sources of the dnaE915, dnaE925, and dnaE947 ant mutator alleles were NR10713, NR11258, and NR10775, respectively (14, 15, 67), using linkage with zac-502−Tn10 or zac-Tn10::cam or both (12). The mutY::Tn10kan and mutM::mini-tet alleles were transferred from strains TT101 (54) and PR86 (63), respectively.

Mutant frequency measurements. For each strain a total of 12 to 18 independent LB cultures (2 ml each) were initiated from single colonies (one colony per tube). The colonies were taken from three to nine independent isolates for each strain. Cultures were grown to saturation at 37°C (or 30°C where indicated) on MM-glucose plates. The number of rifampin-resistant colonies was determined by plating 0.1 ml of a 10−4 dilution on MM-glucose plates (supplemented with tetracyphon when required). The number of lac revertants was determined by plating 0.1 or 0.05 ml of undiluted cultures on MM-lactose plates. The number of trpE9777 revertants was determined by plating 0.1 ml of a 10-fold dilution on MM-glucose plates. The number of rifampin-resistant colonies was determined by plating 0.1 ml of 10-fold-diluted cultures on LB plates containing rifampin (100 μg/ml). Plates were incubated for 20 h (LB) or 40 h (MM) at either 37 or 30°C. To calculate mutant frequencies, the number of mutants per plate was divided by the number of total cells. Occasional jackpot cultures were removed from the analysis. Average frequencies with standard errors (SE) were determined by using the statistical software program Prism (GraphPad).

RESULTS

To further our understanding of the dnaX36 mutator and the mechanism(s) by which the τ subunit may promote fidelity, we studied the interaction of dnaX36 with a series of DNA repair or replication defects. We evaluated the effects on the dnaX36-promoted mutagenesis by using four different mutational assays: reversion of two defined lac alleles (9), reversion of the trpE9777 allele, and acquisition of rifampin resistance (RifR). The two lac alleles permit specific measurement of a G·C→T·A or A·G→T·A transversion using the F′proloc originally present in strains CC104 or CC105 (9). The trpE9777 allele permits measurement of (−1) frameshift errors by loss of an A·T base pair from a run of six A·T pairs (75). Rifampin resistance measures a variety of base substitutions in the rpoB gene (26). Previous studies showed the two indicated lac transversion alleles and the trpE frameshift allele to be particularly sensitive to the dnaX36 mutator effect (61). All of the strains used were mismatch repair deficient (mutL) to facilitate the analysis of mutagenesis in terms of uncorrected replication errors.

Interaction of dnaX36 with the mutM and mutY pathways. We first sought to corroborate our contention that the dnaX36 mutator effect is related to increased production of replication errors rather than any increased DNA damage processing. The mutM and mutY genes play an important role in protecting the cell against the mutagenic effects of 8-oxoguanine.
### TABLE 1. *E. coli* strains used in this study

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<sup>a</sup> F'CC102, F'CC104, F'CC105, and F'CC106 refer to the F′prolac originally present in strains CC102, CC104, CC105, and CC106, which permit measurement of lac G·C→A·T, G·C→T·A, A·T→T·A, and A·T→G·C base substitution mutations, respectively (9). The designations F′CC104/dinB and F′CC105/dinB indicate deletion of the dinB gene on the F′ episome. See Materials and Methods for details on the various constructions.

<sup>b</sup> All of these strains are also ara thl mutL. Δprolac.

(8-oxodG). This base analog is mutagenic due to its ambivalent base-pairing properties and capable of pairing with both C and A during DNA synthesis (74). MutM is a DNA glycosylase capable of removing 8-oxodG from 8-oxodG·dC pairs, whereas MutY is a glycosylase that removes A from 8-oxodG·dA pairs. Lack of either MutM or MutY leads to increased G·C→T·A mutations (53). Since G·C→T·A are among the most frequent mutations induced in a dnaE36 strain (61),
we investigated a possible interaction between the dnaX36 and the mutMmutY pathways. The results in Table 2 (experiment 1) show that the single dnaX, mutM, and mutY defects produce significant mutator effects (74, 3.6-, and 13-fold for the lac G · C→T · A allele). However, the double dnaX36 mutM or dnaX36 mutY strains did not show any major changes (≈2-fold) in the frequency of G · C→T · A transversions compared to the single dnaX36 strain. Thus, it is unlikely that the strong increase in G · C→T · A transversions in the dnaX36 strain is related to the presence of 8-oxodG in the DNA.

The dnaX36 mutator effect is independent of RecA functions. RecA is an essential factor in mediating both homologous recombination and the inducible SOS response. Both aspects of RecA function are involved in modes of mutagenesis, such as adaptive mutagenesis, which requires the RecA recombination function (17), and SOS mutagenesis, which requires both RecA itself and the RecA-regulated error-prone DNA polymerase V (umuDC gene product) (80, 84). The results in Table 2 (experiment 2) indicate that the dnaX36 mutator effect is not obviated by the recA456-null mutation. Although the absolute frequencies in the recA456 strain are reduced by a few fold, a similar reduction is observed for the dnaX36 mutant effect at essentially the same level (60- to 70-fold for G · C→T · A and 15- to 40-fold for A · T→T · A). The reduction in mutant frequencies in recA456 cells may relate to copy number issues of the F episome.

Role of Pol I. To investigate the role of DNA Pol I in the dnaX36 mutator effect, we used the polA1 allele, a defective allele that lacks polymerase activity but still retains the 3′→5′ exonuclease activity (32, 39). ΔpolA alleles have been reported but are generally inviable (32), indicating the important role of Pol I in the cell, likely related to its role in removing the RNA primers that initiate Okazaki fragments. Our experiments revealed that it was not possible to create a dnaX36 polA1 double mutant, even at low temperatures (down to room temperature). This inviability is reminiscent of the observed synthetic lethality of polA1 with a number of other repair/replication defects, such as recA, recBC, uvrB, and others (39). We conclude that dnaX36 strains have an increased requirement for DNA polymerase I, which is consistent with some level of DNA replication defect in the dnaX36(ts) strain (26). On the other hand, a dnaX36 polA1 double mutant could be readily obtained by transduction if the recipient cell contained a plasmid carrying the gene for rat Pol β (79). This result (not shown) is consistent with dnaX36 strains requiring additional DNA polymerizing capacity, possibly because of stalled replication forks and/or an increased number of DNA gaps.

Role of Pol IV and Pol V. E. coli Pol IV and Pol V (the products of the dinB and umuDC genes, respectively) are two accessory polymerases characterized by the lack of 3′ exonuclease (proofreading) activity, and they are generally considered error-prone (83, 86, 87). Both polymerases are expressed in an inducible manner as part of the SOS response. The two polymerases differ in their basal expression levels. Pol IV, when expressed from a single chromosomal gene copy, is present at a level of about 250 copies (35), and this is further increased ~7-fold by SOS induction (36). In contrast, Pol V is considered to be largely absent from normal cells but is strongly induced by the SOS response (92). In view of the error-prone character of these polymerases, we investigated their role in the dnaX36 mutator effect. Considering the lack of effect of the recA deficiency on the dnaX36 mutator effect (see above), any effect of these polymerases would have to be mediated by their basal levels.

We measured the frequency of lac G · C→T · A transversions and of (−1) framen shifts at the trpE9777 locus. In the strains used, the lac gene is located on the F episome (9). Since the pro-lac region on the F episome also includes the dinB gene, the strains used have both chromosomal and episomal copies of dinB. We therefore conducted our experiments with a series of four strains, containing (i) the full set of dinB copies (chromosomal and episomal), (ii) only the episomal copy, (iii) only the chromosomal copy, or (iv) neither copy. The results of the mutagenesis experiments are presented in Fig. 1. It can be seen that in the dnaX36 background both lac and trp reversion strongly depend on dinB (i.e., Pol IV activity). Notably, comparing the fully dinB-deficient strain to the fully proficient one, ~75% of the lac G · C→T · A mutations and 90% of trpE framenhift mutations are dinB dependent. Loss of dinB from the episome causes a stronger effect than loss from the chromosome. This likely reflects the copy number of F, which is generally greater than one. Using quantitative PCR measurements, we estimated the copy number to be ca. two to three in these strains (data not shown), which is
consistent with reports by others (17, 19, 36). Nevertheless, even in the absence of Pol IV the dnaX36 mutator effect for the lac allele is still significant (eightfold), indicating that there are both Pol IV-dependent and Pol IV-independent components to the dnaX36 mutator effect.

Figure 1 also includes the results with the Pol V-deficient strains (umuDC). It is clear that the dnaX36 mutator effect is fully umuDC-independent regardless of the dinB configuration, which is consistent with the results obtained with the recA56 strain.

Role of Pol II. DNA Pol II is a proofreading-proficient polymerase encoded by the polB gene. Like Pol IV, Pol II is an SOS-inducible enzyme with a relatively high basal level (30 to 50 copies per cell), which is increased ~7-fold upon SOS induction (5, 8, 29, 62). Genetic studies have shown that Pol II may be involved in a variety of cellular activities, such as repair of DNA damaged by UV irradiation (48) or oxidation (11), repair of interstrand cross-links (2), lesion bypass (20), replication restart after UV irradiation of E. coli (65), adaptive mutagenesis (17, 18), and long-term survival (93). In vitro
studies have shown that Pol II interacts with Pol III accessory proteins (β-clamp and clamp-loading complex) to become competent to synthesize DNA with high fidelity and processivity (6, 27). Most recently, Pol II was shown to be capable of playing a backup role as a proofreader for Pol III-produced misinsertion errors and to protect mismatched 3’ termini against the action of Pol IV (1).

To investigate the possible role of Pol II in the dnaX36 mutator effect, we used two alleles of polB: ΔpolB and polBex1 (64). The ΔpolB is a simple deletion, whereas polBex1 encodes an error-prone Pol II defective in exonucleolytic proofreading but proficient in DNA synthesis activity (18). This allele is useful because the lack of exonuclease may allow detection of limited amounts of Pol II participation in DNA replication through a mutator effect (1). We also made polB strains that are additionally defective in Pol IV (dinB). Mutagenesis was tested by lac reversion (G·C→T·A and A·T→T·A), trpE9777 reversion, and forward Rifr mutagenesis. The results are shown in the panels of Fig. 2. For the dnaX+ strains, the ΔpolB allele did not significantly affect any of the mutant frequencies, a finding consistent with previous findings on ΔpolB strains (1, 64). In contrast, the polBex1 strains showed increased lac mutagenesis, 12-fold for G·C→T·A and 8-fold for A·T→T·A, although little effect was seen for the trpE or Rifr markers. The mutator effect of polBex1 for the lac alleles parallels that seen by Banach-Orlowska et al. (1), although in that study the lac alleles were located on the chromosome instead of the F’ episome. These results are indicative of a role of Pol II in both chromosomal and episomal DNA synthesis.

In the dnaX36 strains, we observed the expected mutator phenotype for all three tested markers: it was strongest for the lac markers (8- and 11-fold) and the trpE marker (12-fold) but only moderate (2-fold) for the Rifr marker (see also reference 61). Interestingly, in the dnaX36 background, sizable effects were observed for both ΔpolB and polBex. The ΔpolB mutation increased the G·C→T·A and A·T→T·A lac mutant frequency by 5- and 9-fold, respectively. Even stronger effects were found for the polBex1 allele. The G·C→T·A and A·T→T·A lac frequencies were enhanced 16- and 22-fold, respectively, while the frequency of Rifr mutations was increased 5-fold. These results are indicative of a significantly expanded role of Pol II in DNA synthesis in the dnaX36 mutant.

When considering the role of Pol IV in these strains, the results clearly reveal that the enhanced mutator effect of the dnaX36 ΔpolB strain is almost entirely dependent on Pol IV (dinB). Thus, one function of Pol II under these conditions is to prevent access of Pol IV. In contrast, only a small antimutator effect (≤2-fold) was observed in dnaX36 polBex1 strains upon loss of Pol IV (dinB). This is further evidence that the access of Pol IV is limited by the presence of Pol II.

Role of Pol III. Our present results reveal significant polymerase trafficking in dnaX+ and, especially, dnaX36 strains. It was therefore of interest to investigate also the role of Pol III in the dnaX36 mutator effect. We first examined the role of the Pol III proofreading activity, encoded by the dnaQ gene (Pol III ε subunit). We combined dnaX36 with the proofreading-impaired dnaQ49 or dnaQ930 allele, which both provide a strong mutator effect in dnaX+ strains. The dnaQ49 (V96G) mutator activity is temperature dependent and results, at least in part, from defective binding of ε subunit to the polymerase (31, 73, 82). The dnaQ930 (H98Y) allele is a stable allele, and its defect is presumed largely catalytic in nature (81). The dnaX36 dnaQ49 double mutant proved to be more temperature sensitive than the single dnaQ49 mutant, and the experiments were therefore performed at 30°C, at which temperature growth was essentially normal. The results in Table 3 (experiments 1 and 2) show that dnaQ49, dnaQ930, and dnaX36 individually all display strong mutator phenotypes for the two lac reversions. However, in the double dnaX36 dnaQ49 and dnaX36 dnaQ930 mutants the mutant frequency is either not substantially altered from that of the single mutants or, at best, consistent with simple additivity of the single mutator effects. Thus, diminished expression of the Pol III proofreading does not appear to influence the dnaX36 mutator effect.

For the Pol III α subunit, we were unable to obtain combinations of dnaX36 with several dnaE(Ts) mutator alleles due to poor viability. However, we were able to obtain combinations of dnaX36 with three dnaE antimutator alleles: dnaE915, dnaE925, and dnaE941. These dnaE alleles are characterized by their improved fidelity of chromosomal and episomal DNA replication in mismatch repair-deficient and -proficient cells (12, 14, 66–68). It was proposed that their increase in replication accuracy does not result from improved insertion fidelity of the Pol III α subunit but, instead, from improved processing of the insertion errors once made, either by increased proofreading or by increased dissociation of the polymerase from the terminal mismatch (14, 15). In Table 3, experiments 3 and 4, we reproduced the antimutator effects as observed in the dnaX+ background. For this case, we also show the effects for the two lac transitions (G·C→A·T and A·T→G·C), since the antimutator effects are typically largest for transitions (at least in mismatch repair-defective strains) (14, 66). Interestingly, the dnaE alleles do not produce antimutator effects in the dnaX36 background. In fact, in several instances a mutator effect is observed, for example, four- to eightfold for dnaE915 or two- to threefold for the dnaE941 allele. These unusual interactions of dnaX36 with the Pol III proofreading deficiencies (dnaQ) and the dnaE antimutator alleles should be taken into account when addressing the mechanisms responsible for the dnaX36 mutator effect.

**DISCUSSION**

**dnaX36 defect.** In the present study we have further investigated the dnaX36 mutator. The precise defect in the dnaX36 mutant is not known but likely involves one or more aspects of the α subunit interaction within HE (61). The dnaX36 mutation is located in DnaX domain V responsible for interaction with the α subunit (22), and biochemical measurements of the τ–α protein–protein interaction have shown significant impairment in this interaction for several of the domain V mutants (61; C. McHenry, unpublished data). Nevertheless, the in vivo replication defect associated with dnaX36 is likely to be modest. The mutant grows normally at 37°C, and temperature sensitivity is only apparent at temperatures above 43°C on salt-free media (26). Thus, while the α–τ interaction may be altered or weakened under normal conditions (37), it is unlikely that it is abolished altogether, and we presume that increased α–τ dissociations happen only occasionally.
FIG. 2. Effect of Pol II (ΔpolB and polBex1) and Pol IV (dinB) on the dnaX36 mutator activity. The dinB strains lack both chromosomal and episomal gene copies. All strains are also mismatch repair deficient (mutL). Mutant frequencies were determined as described in Materials and Methods. In this series of experiments, the background mutant frequency for the lac G → C → T → A was higher than in previous experiments (Table 2 and Fig. 1). The present experiments were performed at a later time and under slightly different conditions. Nevertheless, within this series of experiments the results were highly consistent and reproducible over several repeats. (A) Mutant frequencies for lac G → C → T → A transversions. The strains used were: NR13153 (dnaX), NR13159 (dnaX dinB), NR16889 (dnaX ΔpolB), NR17225 (dnaX ΔpolB dinB), NR16878 (dnaX polBex), NR17223 (dnaX polBex dinB), NR13256 (dnaX36, polB), NR17225 (dnaX36, polB dinB), NR16878 (dnaX36 polBex), and NR17223 (dnaX36 polBex dinB). The mutant frequencies ± the SE values were 17.2 ± 1.3, 6.3 ± 0.8, 16.8 ± 1.9, 7.9 ± 0.5, 206 ± 13, 119 ± 8, 135 ± 10, 35 ± 5, 643 ± 86, 22 ± 2, 2,210 ± 239, and 1,460 ± 168, respectively. (B) Mutant frequencies for lac A → T → A transversions. The strains used were NR16226 (dnaX), NR16228 (dnaX dinB), NR16890 (dnaX ΔpolB), NR17226 (dnaX ΔpolB dinB), NR16879 (dnaX polBex), NR17224 (dnaX polBex dinB), NR16108 (dnaX36 polBex), and NR16116 (dnaX36 polBex dinB). The frequencies ± the SE were 313 ± 19, 452 ± 57, 515 ± 28, 289 ± 24, 404 ± 37, 309 ± 19, 3,770 ± 350, 403 ± 49, 9,210 ± 2,268, 633 ± 98, 4,790 ± 487, and 4,280 ± 332, respectively. (C) Mutant frequencies for trpE9777 reversion. Strains used were as described in panel A. The mutant frequencies ± the SE were 343 ± 35, 374 ± 13, 484 ± 24, 327 ± 19, 500 ± 63, 345 ± 14, 707 ± 48, 617 ± 44, 714 ± 92, 500 ± 71, 3,800 ± 550, and 2,800 ± 218, respectively. (D) Mutant frequencies for RifR. The strains used were as described in panel A. The mutant frequencies ± the SE were 343 ± 35, 374 ± 13, 484 ± 24, 327 ± 19, 500 ± 63, 345 ± 14, 707 ± 48, 617 ± 44, 714 ± 92, 500 ± 71, 3,800 ± 550, and 2,800 ± 218, respectively.
Our experiments have demonstrated a dependence of *dnaX36* on the DNA synthesis function of Pol I. Since Pol I generally performs gap-filling reactions, this may indicate an increased number of such gaps in *dnaX36* mutants, presumably produced by premature dissociations of Pol III from the primer terminus. On the other hand, we found that *dnaX36* is fully compatible with a recA deficiency. Since a recA defect is synthetically lethal with several other DNA processing defects, such as *dam*, *dat*, *rdhB*, and *polA1* (39, 40), this suggests that the number of DNA interruptions in *dnaX36* is likely limited.

We present below arguments that proper α-τ interaction is particularly important when Pol III is temporarily stalled. Stalling may occur when the polymerase encounters certain obstacles such as DNA lesions, secondary structures, or DNA mismatches. For mutagenesis, the behavior of Pol III at mismatches is likely most relevant. On the other hand, events initiated at terminal mismatches are likely only a subtraction of all events for which the τ-α interaction affects DNA replication. Thus, the fact that the *dnaX36-polA1* combination was not viable should not be construed to indicate that Pol I is involved in the mutagenesis process, although this cannot be excluded. Broadly, the mechanisms responsible for the *dnaX36* mutator effect will be considered within two possible models, which may not be mutually exclusive. In the first, HE may be structurally destabilized in the *dnaX36* mutant and dissociate more frequently from the primer terminus due to the impaired α-τ interaction, providing an opportunity for other DNA polymerases to contribute to the overall error rate. In the second model, the *dnaX* mutator effect results more specifically from loss of a “mismatch management” function that we have previously proposed for τ subunit (61).

**dnaX36** mutator and role of accessory DNA polymerases. An important current finding is the significant role of the accessory polymerases Pol IV and Pol II in the *dnaX36* mutant, either by promoting errors (Pol IV) or by preventing them (Pol II). The intrinsic error-prone potential of Pol IV is well known (35, 41, 83, 88). However, access of Pol IV to the growing point must normally be limited, because the presence or absence of Pol IV does not measurably affect the mutation rate when *dinB* is present as a single chromosomal copy (42, 90). On the other hand, a mutator effect of Pol IV was previously noted for the lacG C→T A allele when *dinB* is present both on F′prolac and the chromosome (about three gene copies total) (42). This effect, noted in the form of an ~2.5-fold reduction in mutation frequency in the fully deficient *dinB* strain, is reproduced in the present study (Fig. 1). However, the effect is specific to the lacG C→T A transversion, and no such effect is observed for the episomal lacA T→C A transversion or the chromosomal *trpE9777* or *prolac* (Rif*) targets. In the *dnaX36* strain, the effect of Pol IV is significantly increased ~4-fold for the G C→T A marker (Fig. 1A and 2A) and is now also observed for the A T→C A transversion and the chromosomal *trpE9777* or *prolac* (Rif*) targets. In the *dnaX36* strain, the effect of Pol IV is significantly increased ~4-fold for the G C→T A marker (Fig. 1A and 2A) and is now also observed for the A T→C A transversion and the chromosomal *trpE9777* or *prolac* (Rif*) targets. In the *dnaX36* strain, the effect of Pol IV is significantly increased ~4-fold for the G C→T A marker (Fig. 1A and 2A) and is now also observed for the A T→C A transversion and the chromosomal *trpE9777* or *prolac* (Rif*) targets. In the *dnaX36* strain, the effect of Pol IV is significantly increased ~4-fold for the G C→T A marker (Fig. 1A and 2A) and is now also observed for the A T→C A transversion and the chromosomal *trpE9777* or *prolac* (Rif*) targets. In the *dnaX36* strain, the effect of Pol IV is significantly increased ~4-fold for the G C→T A marker (Fig. 1A and 2A) and is now also observed for the A T→C A transversion and the chromosomal *trpE9777* or *prolac* (Rif*) targets.
IV competes poorly with Pol II. Furthermore, the very strong dnaX36 polBex1 mutator effect (with or without dinB) indicates that the role of Pol II extends beyond simply excluding Pol IV and that Pol II plays an important role in maintaining fidelity in dnaX36 strains.

Increased or altered DNA polymerase usage in HE has also been reported for certain other E. coli strains bearing mutations in Pol III subunits. Increased usage of Pol IV was reported for the dnaE1336 mutator mutant (76). Likewise, increased usage of Pol IV, as well as an important mutation prevention role for Pol II, were observed for the dnaE486 and dnaE511 mutator mutants (1, 41). The dnaN159 mutant, carrying a defective β-clamp was also reported to display altered polymerase usage (78). The dnaN159 mutant, like the dnaX36 mutant, is also dependent on the Pol I polymerase activity for viability (78).

**Initial error production by accessory polymerases?** Of critical importance regarding the role of Pol III, and of other polymerases, in the dnaX36 mutator effect is the origin of the errors that lead to the observed mutations. One simple possibility to account for the role of the accessory polymerases would be that during replication Pol III becomes occasionally disengaged from the replication point, and synthesis is then continued by accessory polymerases. This may lead to increased error production when synthesis is performed by an error-prone enzyme such as Pol IV or the exonuclease-deficient form of Pol II. Within such a model, the dnaX36 mutant simply suffers from increased Pol III disengagement and, hence, increased error production. This mode of mutagenesis is certainly consistent with the decrease in mutagenesis in dnaX36 dinB strains and the increase in mutagenesis in dnaX36 polBex1 strains. It is also consistent with the lack of effects of the dnaQ alleles on dnaX36 since, within this model, the intrinsic Pol III error rate would be irrelevant. Nevertheless, even in the absence of Pol IV (dnaX36 dinB strains) there is still a significant residual dnaX36 mutator effect, at least for the lac G·C→T·A mutations (~8-fold, Fig. 1A). A strong dnaX36 mutator effect is also observed in an alternative experimental system where the lac operon is located chromosomally (15) and dinB is present in only one copy; under these conditions the dnaX36 mutator effect is not dinB dependent (results to be published elsewhere). Since Pol V is likewise not required (Table 2 and Fig. 1), ongoing DNA synthesis by error-prone polymerases is not an obligatory feature of the dnaX36 mutator effect. A second concern with this kind of model is that an accounting for observed mutator effects through DNA synthesis by error-prone polymerases requires extensive amounts of synthesis by such enzymes that may not seem realistic (1, 13, 42, 47). For example, assuming that the exonuclease-deficient form of Pol II is roughly 100-fold less accurate than Pol III HE (7), the 100-fold or more mutator effect of the dnaX36 PolBex1 strains for the lac alleles (Fig. 2A,B) would require Pol II to synthesize essentially the entire chromosome. Lower but still significant numbers would apply to error-prone synthesis by Pol IV, which is essentially a low-processivity enzyme.

**Initial error production by Pol III.** In contrast to the random Pol III dissociations considered above, we consider terminal mispairs created by Pol III misinsertion errors as the more relevant events for the role of τ subunit and the accessory polymerases (1, 13, 42, 47). Terminal mispairs are known impediments for ongoing synthesis (33, 52, 58) and constitute potential stalling points. At such sites, polymerases face a choice among several competing paths, including direct extension from the mismatched primer, transfer of the mismatch to the exonuclease site, or dissociation from the mismatch (with concomitant opportunity for access by other polymerases). The proportioning among these pathways is a major determinant for the ultimate error rate, and τ, as the central, coordinating subunit of HE, is likely a determinant of this proportioning.

In Fig. 3, we diagram several of the possible polymerase pathways at a terminal mismatch created by Pol III. If HE were to continue synthesis from this mismatch a mutation would result (at least in the absence of mismatch repair, as is the case in our study) (line 2). Of course, the by far more likely outcome is removal of the mismatch by the HE proofreading activity (top line). As a third option (line 3), the polymerase may dissociate from the mismatch. This dissociation, permitting access of other DNA polymerases to the growing point, is likely to be rare in dnaX+ strains but may occur with increased probability in a dnaX36 mutant. Binding of the abandoned mismatch by either Pol II or Pol III (or HE) would lead to removal of the mismatch by their exonuclease activities, but binding and extension by Pol IV would be mutagenic. This scenario of enhanced dissociation at terminal mismatches due to increased instability of HE in the dnaX36 mutant may provide one possible explanation for its mutator effect, while also accounting for the significant role of Pol IV. An alternative model is described below.

**A mismatch-management function for τ?** One problem with the straightforward dissociation model described above is that all dnaX36-induced mutations necessarily result from the action of Pol IV. In the present system this may be the case for the trpE9777 framen shift mutations but not for the lac G·C→T·A transversions for which a significant dinB-independent component is noted (Fig. 1 and 2). For example, in the completely dinB-deficient lines, the dnaX36 mutator effect is still 8-fold (11.8 versus 1.4) compared to the 13-fold effect (45.7 versus 3.4) in the fully dinB-proficient strain (Fig. 1A). Thus, both Pol IV-dependent and -independent components exist.

The alternative analysis follows from a previously described model in which τ was proposed to function as a sensor for HE molecules stalled “nonproductively” at certain terminal mismatches, and in which the observed mutator effect of dnaX36 and related domain V dnaX mutants resulted from the lack of this τ sensor function (61). The τ sensor function may be considered analogous to the described function of τ in the lagging strand, where τ is capable of sensing that Pol III has reached the end of an Okazaki fragment (45, 46, 51), which triggers the release of Pol III from the primer terminus, enabling HE to resume synthesis at the next Okazaki fragment. Indirect evidence for stalling of HE at terminal mismatches has been obtained from in vitro fidelity studies on HE (59, 60). The stalled HE complexes were termed “nonproductive” since neither extension nor proofreading of the mismatch appeared to take place for long periods of time (59, 60). It was proposed that the resolution of such stalled complexes, if occurring in vivo, would require the sensing action of τ (61). Resolution might occur in the simplest way by enabling the conformational switch to the Pol III exonuclease mode. Alternatively, resolu-
tion could involve dissociation of the nonproductive Pol III from the mismatch in favor of another proofreading polymerase, such as Pol II. Rebinding of the mismatch by Pol III itself would also lead to removal of the mismatch, since initial binding to a primer terminus favors the exonuclease site (30, 60). Evidence has been presented for a possible tool-belt organization of Pol III HE, in which a secondary site on the β-clamp accommodates an additional polymerase capable of switching places with Pol III (28). Furthermore, the recent discovery that HE might contain three subunits and three Pol III core modules (50) would allow for a convenient shuttling of the mismatch to the additional Pol III unit, followed by proofreading. It seems reasonable to assume that these various polymerase switches would be facilitated by the action of HE.

The various options emanating from a stalled Pol III complex are outlined in the bottom section of Fig. 3. Line 6 represents the normal HE-mediated mode of error removal as described above. However, in the dnaX36 mutant, due to impairment of the τ-α interaction, this resolution option may not be operative. One consequence might then be that Pol III is ultimately forced to extend the mismatch, fixing the mutation (line 5). This mode of mismatch extension provides a sufficient explanation for the dinB-independent component of the dnaX36 mutator effect observed in the present study (Fig. 2 and 3). Alternatively, Pol III may ultimately abandon the mismatch even without any assistance of HE (line 4). This (possibly less controlled) dissociation may provide an avenue for Pol IV to increase the error rate, when present in sufficiently large amounts, or for Pol II to remove the mismatch, which appears to be the predominant event.

This model for the dnaX36 mutator effect is also supported by its unusual specificity, namely, the specific enhancement of transversions and −1 frameshifts at “non-run” sequences (61). Since transversion mismatches (purine·purine or pyrimidine·pyrimidine) are structurally the most distorting and the most difficult to extend (33, 52), these mismatches are the ones most likely to lead to a stalled HE complex. Hence, forced extension of these mismatches yields transversion base substitutions or, in a permissive sequence context, frameshifts by realignment of the mismatched primer base on the next (correct) template base. The latter option, when permitted by the sequence context, is strongly favored by HE over direct extension (55, 59–61).

A hierarchy of polymerases. One significant observation from the present experiments is the exceptionally large role that Pol II appears to play in the maintenance of fidelity in the dnaX36 mutant. Pol II competes effectively, of course, against Pol IV, but the overall fraction of errors removed by Pol II is particularly notable. For example, for the lac G·C→T·A reporter, the polBex1 allele increases the mutant frequency of the dnaX36 strain by 16-fold (dinB) and 42-fold (dinB) (Fig. 2A), which, within the context of the model of Fig. 3, may be interpreted to mean that in the polB+ background 93 to 98% of available Pol III errors will be corrected by Pol II. Similar numbers apply to the lac A·T→T·A allele (Fig. 2B) and the Rif+ mutants (Fig. 2D). One (partial) exception is the case of the trpE9777 frameshift mutations, which are affected significantly (10-fold) by the polBex1 allele only in the dinB background. It is possible that the slipped primer-template intermediate(s) that underlie the frameshift mutations are particularly amenable for processing by Pol IV. It should also be noted that in most cases the effect of a polB deletion is not as pronounced as that of polBex1 (Fig. 2A, B, and D). In the ΔpolB background dnaX36-mediated mutagenesis is enhanced.

FIG. 3. Diagram depicting the various ways by which a Pol III misinsertion error may be processed in an error-free or error-prone manner. The scheme includes the competition between polymerases that may ensue upon dissociation of a Pol III HE from the mismatch (line 3 or 4), as well as the proposed τ-dependent mode of error removal (61) (line 6). Note that Pol III, while initially unable to proofread the error in the stalled state, may do so effectively upon rebinding, since the latter may occur preferentially using the exonuclease active site (30, 59, 60).
(in a Pol IV-dependent manner), but this increase is not as large as in the polB helix background. One might conclude that, in the absence of Pol II, Pol III (or HE itself) is also capable of competing effectively with Pol IV. Thus, a hierarchy of DNA polymerases in addressing abandoned terminal mispairs may be Pol II > Pol III > Pol IV.

Role of proofreading. Of interest regarding the dnaX36 mutator effect is the apparently lack of a role of the Pol III proofreading. Within the context of a simple dissociation mechanism (Fig. 3, line 3) one might argue that lack of Pol III proofreading would increase the number of mismatches available for dissociation but would not fundamentally alter the chance of Pol IV to access the mismatch once dissociation occurred; therefore, the lack of proofreading would not alter the magnitude of the mutator effect (fold increase). As our experiments with the dnaQ mutations indicate, this is not what is observed; instead, the dnaX mutator effect is either no longer apparent or at best additive with the dnaQ mutator effect (Table 3). Thus, this result might be used to argue against the simple dissociation model. Alternatively, one may argue that in proofreading-impaired backgrounds the dissociation-mediated mutator pathway (Pol IV effect) can become obscured. More precisely, in a limit case of “no proofreading” the flow of mutations through the direct extension pathway (line 2) may be high relative to that through the dissociation pathway (line 3), whereas the latter is ultimately limited by the ability of Pol IV to compete with Pol II and Pol III. An answer to this question would require detailed insight into the precise rate constants of each of the reactions in Fig. 3.

The proofreading observations are more simply explained based on the stalled complex model (lines 4 to 6). The stalled complex is characterized a priori as a nonextending and nonproofreading entity (61). Thus, the flow of mutations through pathways 4 and 5 might be largely independent of the Pol III proofreading. Second, at any time only a fraction of terminal mismatches is expected to result in a stalled complex. Therefore, any proofreading occurring in lines 4 to 6 would run in parallel to the conventional main proofreading pathway (line 1). Therefore, simple additivity of effects may sometimes be expected (Table 3). Finally, in the case of the dnaE “antimutators” (Table 3), it is likely that some of these dnaE alleles, in addition to increasing proofreading through the conventional pathway (an antimutator effect), are also, due to their catalytic impairment, more likely to end up in a stalled state. They may thus be expected to enhance in some cases the dnaX36 mutator effect.

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